(12) UK Patent Application (19) GB (11) 2 051 357 A

(21) Application No 8008095
(22) Date of filing 11 Mar 1980
(30) Priority data
(31) 48319U
(32) 14 Jun 1979
(33) United States of America (US)
(43) Application published 14 Jan 1981
(51) INT CL³ G01N 33/54
(52) Domestic classification G1B BA BR
(56) Documents cited
GB 2010480A
GB 1562804
GB 1561042
GB 1443181
(58) Field of search G1B
(71) Applicant
Abbott Laboratories, 14th Street and Sheridan Road, North Chicago, Illinois 60064, United States of America
(72) Inventors
Chung-mie Ling,
Richard A. Decker,
Lacy R. Overby
(74) Agent
Lloyd, Wise, Tregear & Co.

(54) **Simultaneous Detection of Indicators of Hepatitis Virus Exposure**

(57) A method for simultaneously detecting in a sample at least two different markers (a marker is an antigen or antibody) evidencing exposure to hepatitis virus comprises contacting the sample with a solid phase reagent which is coated with at least two different, non-complementary immunoreactants which are complementary to the unknown markers to be detected, then with a liquid reagent comprising at least two different hepatitis markers or immunoreactants, each selected to either react or compete with one of the unknown markers and each labeled with a detectably distinct tag.

Form 892
References

GB 2 051 357A

SPECIFICATION

Method and Reagents for Simultaneously Detecting Different Markers of Hepatitis

This invention discloses an improvement in solid phase immunoassay methods for the detection and determination of antigens and antibodies (markers) relating to hepatitis.

There are at least two distinct types of viral hepatitis. Hepatitis A is believed to be caused by the hepatitis A antigen (HAVAg) and is generally characterized by an incubation period of two to six weeks, mild prodromata and a relatively mild clinical illness. The disease is generally transmitted by contaminated food or liquid, but has also been shown to be transmitted by systemic inoculation. Hepatitis A is frequently called "infectious hepatitis", and in the United States the number of reported cases is over 50,000 annually.

The hepatitis B virus is believed to be the most probable etiologic agent for "serum hepatitis". Hepatitis B infection is generally transmitted by blood products or contaminated instruments such as needles, but it may also be transmitted by other means. Previously, a hepatitis B infection was associated with an incubation period ranging from six weeks to six months. However, incubation periods as short as two weeks have been documented. The illness may be mild or asymptomatic, but if symptomatic, manifestations may be especially severe. Prodromata may include arthralgias, arthritis, rash, fever, anorexia, fatigue and pruritis with or without jaundice.

At least three distinct antigen-antibody systems can be associated with hepatitis virus B: the surface (HB_sAg: anti-HB_s), the "core" (HB_cAg: anti-HB_c), and e-antigen (HB_eag: anti-HB_e). The hepatitis B surface antigen (HB_sAg) is found in the blood as 22 nm spheres and as elongated tubules which are 22 nm in diameter and variable in length and is believed to represent the protein coat of the hepatitis B virus.

A 42 nm particle containing DNA and a DNA polymerase is considered to represent the infectious virus (Dane particle). The surface of the Dane particle is similar or identical to HB_sAg. In detergents, the Dane particle is degraded to a 27 nm electron dense core, HB_cAg. The latter is seen in nuclei of hepatocytes of patients with serum hepatitis during the acute infection stage.

Thus, patients with viral hepatitis type B might be expected to produce antibodies to the surface antigen (anti-HB_s), and also to the protein core (anti-HB_c). HB_sAg in serum has been a consistent marker for ths presence of the hepatitis B virus, and anti-HB_c usually appears during early viremia, often accompanying antigenemia (HB_sAg), at the height of liver disfunction and long before the appearance of anti-HB_s. Anti-HB_c is generally associated with prolonged circulation of HB_sAg suggesting that anti-HB_c is produced in response to the active replication of the virus.

In 1972, the hepatitis e antigen (HB_eAg) was detected and characterized. The e marker has been found in HB_sAg-positive serum and is thought to occur more commonly in serum of chronic HB_sAg carriers with active liver disease than in healthy carriers. In patients, during the incubation period of hepatitis B, the e antigen was shown to appear just after the appearance of HB_sAg and before clinically apparent liver injury. Logically, its presence in such sera would be indicative of a poor prognosis and on-going liver damage. Conversely, the presence of e antibody (anti-HB_e) would be indicative of a good prognosis. These correlations are not absolute, but may be useful clinical guides.

Since the serologic markers for hepatitis appear in consistent, sequential order during the course of infection, acute disease and recovery, an analysis for two or more markers would be valuable to assess the time-course of the disease.

An assay for more than one marker can also provide an important double-check for HB_sAg in blood doners or patients in an effort to reduce the incidence of type-B hepatitis. Such a need has been demonstrated by Goldfield *et al*; Am. J. Med. Sci., 270: 335—342 (1975), who in careful follow-ups of recipients of blood negative for HB_sAg, found evidence of exposure to the hepatitis antigen in 7 of 465 patients. Clearly, a method of detecting more than one marker to the hepatitis B virus should decrease or prevent the occurrence of false negatives. Similarly, in many instances a positive response to two tests minimizes the possibility of a false positive occurring in one test.

While the reagents and methods described and claimed herein are similar to known commercial products and procedures for detecting the various markers to viral hepatitis, heretofore there has been no disclosure or suggestion describing any techniques for detecting said markers simultaneously.

Accordingly, this specification describes in detail reagents and a method useful for detecting simultaneously in a sample at least two different markers evidencing exposure to hepatitis virus.

The method comprises contacting the sample with a solid support which has been coated with at least two different, non-complimentary immunoreactants which are complimentary to the unknown markers; incubating the sample with the coated solid support for a period up to 24 hours;

separating the coated solid support from the sample;

washing the coated solid support;

contacting the washed solid support with a liquid reagent comprising at least two different hepatitis markers or immunoreactants, each selected to either react or compete with one of the unknown hepatitis markers and each labeled with detectably distinct tags;

separating the solid phase from the liquid reagent; and

determining the presence of labeled markers on the solid support by detecting each distinct tag.

The following examples will demonstrate the

preparation and use of representative reagents within the scope of the claimed invention. The first example describes the method of preparing a solid support coated with two different non-complimentary immunoreactants which are complimentary to markers evidencing exposure to hepatitis.

More specifically, a solid support (bead) has been coated with two different, noncomplimentary immunoreactants in such a manner that both retain their reactivity and are able to combine with complimentary markers in an unknown specimen.

In the first example a polystyrene bead has been coated with an antibody to HB_sAg and with the core antigen, HB_cAg. The antibody retains its avidity and will react with any HB_sAg in the unknown specimen and the affixed core antigen will retain its antigenicity and react with any antibody to the core (anti-HB_c) in the unknown specimen. The crux of this invention is that both immunoreactions will occur simultaneously with a single solid phase reagent.

Example I

Preparation of Coated Support

A formulation of HB_cAg was made by exposing Dane particles to a solution of 2% 2-mercaptoethanol (2-ME) and 5% Tween 80 in Tris-EDTA-saline (TSE) buffers at 37°C for 2 hours. That solution was diluted 10-fold in 5% 2ME-TSE and allowed to stand overnight before diluting to final concentration of 1:8000 in TSE. Separately, polystyrene beads (1/4") were coated with a 1:2000 dilution of anti-HB_s serum (guinea pig) in phosphate buffered saline (PBS) by soaking for two hours. The beads were removed, washed and dried. The core preparation was then poured over the coated beads and the core antigen was allowed to adhere to the bead for 2 days at room temperature. The beads were removed from the buffer solution, washed, coated with a 10% sucrose solution to stabilize the adherents and air dried.

Although polystyrene beads are preferred because they are easily coated and manipulated, any solid support of either macro or micro dimensions, fashioned from a variety of plastics, metal and glass could just as easily be coated and used to demonstrate the claimed invention.

While it is possible that the beads may be coated first with core antigen and subsequently coated with anti-HB_s, it has been observed that coating the beads first with the guinea pig anti-HB_s serum significantly augments the adherence of core antigen.

It is also important to note that other combinations of non-complimentary immunoreactants relating to hepatitis virus may also be employed. The immunoreactants must, of course, be non-complimentary because a complimentary attraction would diminish the antigenicity and avidity of the reagent.

The following example will demonstrate the preparation of a liquid phase reagent containing an immunoreactant, anti-HB_s, tagged with a radio label (^{125}I) and a marker, anti-HB_c, tagged with a reactive enzyme label (horseradish peroxidase).

While the terms "marker" and "immunoreactant" might be used interchangably, it is preferable if the term "marker" is used to designate the antigen or antibody, and their equivalents, to be detected in the unknown sample. The term "immunoreactant" is used to define either the antigen or antibody complimentary to the markers to be detected.

Accordingly, in the following examples, the antigen and antibody employed on the solid support are always complementary to one of the unknown markers, and, therefore, are referred to as immunoreactants.

In the liquid phase reagent, the labeled surface antibody is complimentary to the unknown marker (HB_sAg) and is, therefore, referred to as the labeled immunoreactant. The labeled core antibody, however, is identical to the marker to be detected and is, therefore, referred to as a labeled marker.

Example II

Tagged Antibody Reagent

The iodination (^{125}I) of antibody to HB_sAg (anti-HB_s) entailed adding approximately 0.1 ml of a 0.5 M phosphate buffer at pH7.5, a small volume of 5—6 mci of $Na^{125}I$, and 100 mg. of purified anti-HB_s to a one dram vial, adjusting the pH to 7.5—8.0 and mixing all ingredients. To this mixture was added 50 microliters of a freshly prepared solution of chloramine T (35 mg in 10 ml. 0.05 M phosphate buffer, pH 7.5). After additional mixing, the reaction was allowed to proceed at room temperature for 60 seconds. Fifty microliters of a freshly prepared solution of sodium metalsulfite (35 mg in 10 ml of 0.05 M phosphate buffer at pH 7.5) were added to the reaction to reduce the chloramine T and thereby stop the reaction.

The reaction efficiency was checked by placing 5 microliters of the reaction mixture on a strip of Whatman No. 1 paper strip and chromatographing the mixture in 70% methanol. The iodinated protein remained at the origin while the free ^{125}I migrated with the solvent. The % of ^{125}I in protein is considered to be the % of reaction efficiency.

The crude iodinated antibody was purified using a Sephadex G-50 gel column with 0.1 molar Tris buffer containing 0.9% sodium chloride at pH 7.8. The column was pretreated with a small volume of a 30% aqueous solution of bovine serum albumin followed by an equal additional volume of 0.1 molar Tris buffer solution containing 0.9% sodium chloride at pH 7.8. The iodinated antibody was added to the top of the column and washed through using the saline buffered Tris solution. The labeled antibody was the first eluate collected from the column.

The conjugation of antibody to hepatitis B core to horseradish peroxidase (HRP) entailed activating peroxidase by using sodium meta

periodate. The excess periodate and by-products were separated from the active peroxidase by gel filtration (sephadex G-25) column. The activated peroxidase was then reacted with the antibody (anti-HB_c). The reaction occurred spontaneously. Sodium borohydride was then added to stabilize the bond formed between peroxidase and the antibody, and acetone was added to destroy the remaining sodium borohydride.

When employed according to the teaching of this invention, the two tagged antibodies are diluted into a diluent containing:

50% fetal calf serum
15% normal human serum
.005 M EDTA
0.1% Tween-20
0.01% Thiomersal in PBS

It should be apparent that a variety of detectable tags may be employed. The only requirement, naturally, is that they be detectably distinct. Any of a variety of isotopes could be utilized just as easily as ^{125}I. It is not necessary that one marker or immunoreactant be radio-labeled and the other enzymatically or florescently labeled since different isotopes are, themselves, detectably distinct. Similarly, all labeled components of the reagent might just as easily employ different enzymes requiring different substrates yielding detectably distinct reaction products.

It should be noted that any of the antibodies and antigens of hepatitis A and B could be labeled to perform according to the disclosed invention. The only immunochemical requirements are that the labeled components not be complimentary with each other or to the markers to be determined.

Example III

Simultaneous Detection of HB_sAg and Anti-HB_c

Serum samples containing the "unknown" hepatitis markers were added to the individual wells of a reaction tray and a polystyrene bead coated in accordance with Example I was added to each specimen sample and allowed to incubate for 2 hours at 45°C. The beads were removed from the reaction well, washed with water to remove any excess reagent, and added to 0.2 ml of the liquid phase reagent prepared in accordance with Example II. The solid and liquid phases were incubated for a period of one hour at 45°C. The solid phase was then separated from the antibody reagent and washed four times with water to remove excess reagent. The washed beads were placed in test tubes containing o-phenylenediamine (30 mg in 10 ml of 0.1 M citrate buffer, pH 5.5) and allowed to incubate for 30 minutes. Following that period the enzyme reaction was stopped by the addition of 1 ml of 1 M HCl and the tubes were visually examined for the presence or absence of color resulting from the reaction of the enzyme tag and the substrate. The absence or low incidence of color indicated that anti-HB_c was present in the unknown sample and competed with the labeled marker for reaction sites on the coated support. Next, radio-activity on the beads was counted in a gamma counter and the CPM's was recorded. Radioactivity on the bead indicated that there was HB_sAg in the unknown sample which adhered to the affixed antibody and provided a binding site for the radio-labeled antibody.

Claims

1. A method of simultaneously detecting in a sample at least two different markers evidencing exposure to hepatitis virus, said method comprising:

a) contacting the sample with a solid phase coated with at least two different, noncomplimentary immunoreactants complimentary to the unknown markers;

b) incubating the sample with the coated solid phase for a period of 1-24 hours;

c) separating the coated solid phase from the sample;

d) washing the coated solid phase to remove unbound sample;

e) contacting the washed solid support with a liquid reagent comprising at least two different hepatitis markers or immunoreactants, each selected to either react or compete with one of the known hepatitis markers, and each labeled with detectably distinct tags;

f) separating the solid phase from the labeled reagent;

g) and determining the presence of labeled markers or immunoreactants on the solid support by detecting the distinct tags.

2. A method of simultaneously detecting in a sample hepatitis B surface antigen and antibody to hepatitis B core antigen, said method comprising:

a) contacting the sample with a solid phase coated with both hepatitis B core antigen and an antibody to hepatitis B surface antigen;

b) incubating the sample with the coated solid phase for a period of 1—24 hours;

c) separating the coated solid phase from the sample;

d) washing the coated solid phase to remove unbound sample;

e) contacting the washed solid support with a liquid reagent comprising antibodies to hepatitis B surface antigen and hepatitis B core antigen each labeled with detectably distinct tags.

f) separating the solid phase from the labeled antibody reagent; and

g) determining the presence of labeled antibodies on the solid support by detecting the distinct tags.

3. The method according to Claim 2 wherein the antibody to the surface antigen is radiolabeled and the antibody to the core antigen is labeled with an enzyme.

4. The method according to Claim 3 wherein the radiolabel is ^{125}I and the enzyme is horseradish peroxidase.

5. A reagent useful for simultaneously

detecting in a sample at least two different markers evidencing exposure to hepatitis virus said reagent comprising:

a solid support coated with at least two different non-complimentary immunoreactants complimentary to the unknown markers.

6. A reagent according to Claim 5 wherein the solid support is further defined as being coated with hepatitis B core antigen and an antibody to hepatitis B surface antigen.

7. A reagent useful for simultaneously detecting in a sample at least two different markers evidencing exposure to hepatitis virus said reagent comprising:

at least two different hepatitis markers or immunoreactants each selected to either react or compete with an unknown hepatitis marker and each labeled with detectably distinct tags.

8. A reagent according to Claim 7 wherein the reagent is further defined as comprising antibodies to hepatitis B surface antigen and hepatitis B core antigen.

9. A reagent according to Claim 8 wherein the antibody to hepatitis B surface antigen is radiolabeled.

10. A reagent according to Claim 9 wherein the radiolabel is ^{125}I.

11. A reagent according to Claim 8 wherein the antibody to hepatitis B core antigen is labeled with an enzyme.

12. A reagent according to Claim 11 wherein the enzyme label is horseradish peroxidase.

13. A reagent according to claim 5, prepared according to any one of the Examples herein.

Printed for Her Majesty's Stationery Office by the Courier Press, Leamington Spa, 1981. Published by the Patent Office, 25 Southampton Buildings, London, WC2A 1AY, from which copies may be obtained.